Proc. Natl. Acad. Sci. USA
Vol. 73, No. 6, pp. 1945–1949, June 1976
Biochemistry

Structure of a nicked DNA–protein complex isolated from simian virus 40: Covalent attachment of the protein to DNA and nick specificity

(protein–DNA bonding/electron microscopy/iodination of proteins)

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Communicated by Jerome Vinograd, April 5, 1976

ABSTRACT **A portion of the nicked circular DNA isolated from purified simian virus 40 contains a protein–DNA complex in which protein(s) is covalently attached to the end of a DNA single strand. (Nicked DNA is double-stranded DNA that contains at least one single-strand scission.) The protein was visualized by electron microscopy and labeled *in vitro* with ^{125}I. The bond between the protein and the DNA is stable in alkali, 4 M guanidine·hydrochloride, 3.86 M hydroxylamine (pH 4.23), and in 98% formamide. Most of the molecules in the nicked circular DNA fraction contained one nick. The nick occurs on either of the two complementary strands; the specific nick sites on the two strands are staggered, but lie within a few hundred nucleotides of each other.**

We have reported previously (1) that a protein–DNA complex is found in the nicked circular DNA fraction when DNA is isolated from purified simian virus 40 (SV40) in the presence of sodium dodecyl sulfate ($NaDodSO_4$) and dithiothreitol. (Nicked DNA is double-stranded DNA that contains at least one single-strand scission.) The protein in the complex remains associated with the DNA at high concentrations of salt and $NaDodSO_4$. It was visualized by electron microscopy (EM) on circular molecules and on molecules cleaved by restriction endonucleases. The protein occurs at 0.67 map unit on the SV40 DNA molecule. In this paper we report that the protein–DNA complex is also stable in the presence of alkali, 4 M guanidine·HCl, 3.86 M hydroxylamine (pH 4.23), and in 98% formamide. Based on the stability in the presence of these reagents, the protein–DNA complex is regarded as covalently bonded. We also report that a single nick occurs in the circular DNA, but on either of the two complementary strands. The position of the nick in one strand is staggered with respect to the position in the other strand. The above conclusion is based on the appearance in the electron microscope of the DNA forms after self-annealing and partial denaturation of the self-annealed molecules.

MATERIALS AND METHODS

SV40 was purified from infected TC-7 cells as described (1), except for the use of a CsCl step gradient purification before the final banding of virus. Band velocity sedimentation was performed by layering the sample, after 2-fold dilution by TD buffer (0.14 M NaCl–5 mM KCl–0.7 mM Na_2HPO_4–2.5 mM Tris·HCl, pH 7.5) of virus in CsCl (ρ = 1.35 g/cm^3), onto a 4-ml step gradient of CsCl (1.0 ml each of CsCl 1.25 g/cm^3, 1.34 g/cm^3, 1.4 g/cm^3, and 1.6 g/cm^3 all in TD buffer) and centrifugation for 2 hr at 33,000 rpm at 20° in an SW50.1 rotor. Viruses were stored in TD buffer at 4° or at −20°.

Viruses (10 mg) were deproteinized in 5% $NaDodSO_4$–5 mM EDTA–5 mM dithiothreitol-TD buffer as described (1). The protein–DNA complex was isolated in a 15–30% linear sucrose gradient containing 0.5% $NaDodSO_4$–1 mM EDTA–10 mM Tris·HCl, pH 7.5. The isolated slowly sedimenting material, containing the protein–DNA complex, was labeled with ^{125}I in 0.1% $NaDodSO_4$–0.1 M borate buffer, pH 8.5 as described by Bolton and Hunter (2). After extensive dialysis in 1% $NaDodSO_4$–4 mM EDTA–10 mM Tris·HCl, pH 7.5 at room temperature, the labeled material was resedimented in a 15–30% linear sucrose gradient. Fractions of 0.8 ml were collected from the bottom and examined for absorbance at 260 nm and for ^{125}I radioactivity. The DNA in the gradient was collected by ethanol precipitation, resuspended in 10 mM Tris·HCl, pH 7.5–1 mM EDTA, and used for the studies reported below. Labeled SV40 DNA was prepared by adding [^{14}C]dThd (0.017 μCi/ml) to the infected cells 20 hr after infection; DNA was isolated by the Hirt procedure (3) 75 hr after infection. Nicked circular SV40 [^{14}C]DNA was prepared by x-irradiation and fractionation in a CsCl gradient containing ethidium bromide (4). PM2 [^{3}H]DNA was a kind gift of Mr. R. Watson.

In order to visualize proteins attached to single-stranded DNA (ss-DNA) by EM, we modified the benzyldimethylalkylammonium chloride (BAC) spreading method from that described by Vollenweider *et al.* (5). Immediately before spreading, 0.5 μl of 2 M triethanolamine (pH 8.7), 0.5 μl of DNA solution (50 μg/ml), and 1 μl of 0.25% BAC stock solution prepared from Zephiran (Winthrop Inc.) in formamide, were added in that order to 98 μl of formamide. Twenty-five microliters of this solution were spread onto a hypophase of redistilled water. The DNA film was picked up on carbon-coated grids, dehydrated in ethanol, stained with uranyl acetate, and shadowed with Pt.

RESULTS

Stability of the virion DNA–protein complex to various chemical agents

We wished to test the stability of the protein-nucleic acid bond to alkali and to high concentration of guanidine·HCl.

^{125}I-Labeled DNA–protein complex, prepared as described in *Materials and Methods*, was sedimented through an alkaline sucrose gradient (Fig. 1a). Thirty-five percent of the ^{125}I radioactivity cosedimented with unit-length linear ss-DNA. SV40 [^{14}C]DNA, nicked by x-irradiation, resolved into two peaks corresponding to circular (18 S) and linear (16 S) ss-DNAs under this condition (Fig. 1b). The DNAs in the pooled fractions (Fig. 1a, fractions 14 through 22) were collected; portions were

Abbreviations: SV40, simian virus 40; EM, electron microscopy; ss-DNA, single-stranded DNA; ds-DNA, double-stranded DNA; nicked DNA, ds-DNA that contains at least one single-strand scission; TD buffer, 0.14 M NaCl–5 mM KCl–0.7 mM Na_2HPO_4–25 mM Tris·HCl, pH 7.5; $NaDodSO_4$, sodium dodecyl sulfate; BAC, benzyldimethylalkylammonium chloride.

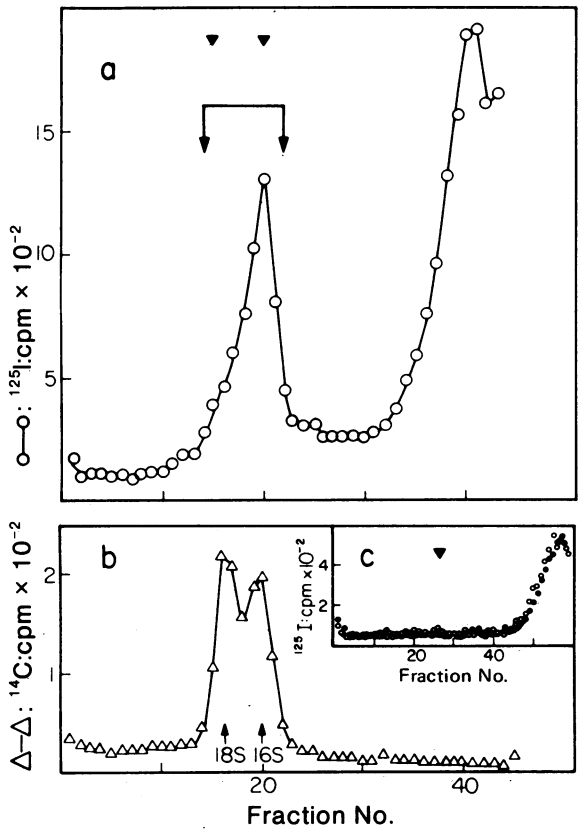

FIG. 1. The effect of Pronase and DNase treatment of the protein-DNA complex on the sedimentation profiles in alkaline sucrose gradients. (a) ^{125}I-labeled complex DNA *in vitro*, 60 μg. (b) X-ray nicked SV40 [^{14}C]DNA. (c) Fractions 14–22, in (a) were pooled; the DNA was precipitated with ethanol, redissolved, treated with enzyme(s), and sedimented again. Superimposed profiles of enzyme-treated samples: (○) Pronase-treated sample, Pronase (Calbiochem; 1 mg/ml) at room temperature for 3 hr in 10 mM Tris·HCl, pH 7.5–0.15 M NaCl–1 mM EDTA; (●) DNase-I-treated sample, DNase I (Worthington Biochemical Corp., 100 μg/ml) at 37° for 1 hr in 10 mM Tris·HCl, pH 7.5–50 mM NaCl–8 mM $MgCl_2$–0.5 mM EDTA. All samples were treated in 0.25 M NaOH at room temperature for 10 min, then sedimented in 5–20% alkaline sucrose gradients containing 0.9 M NaCl–0.1 M NaOH–0.001 M EDTA–0.015% Sarkosyl at 20° for 6 hr at 36,000 rpm in an SW50.1 rotor. Fractions were collected from the bottom of the tubes. The triangles at the top of (a) and (c) indicate the fractions taken for EM analyses (Table 1). Recovery of input ^{125}I radioactivity was 65–75%.

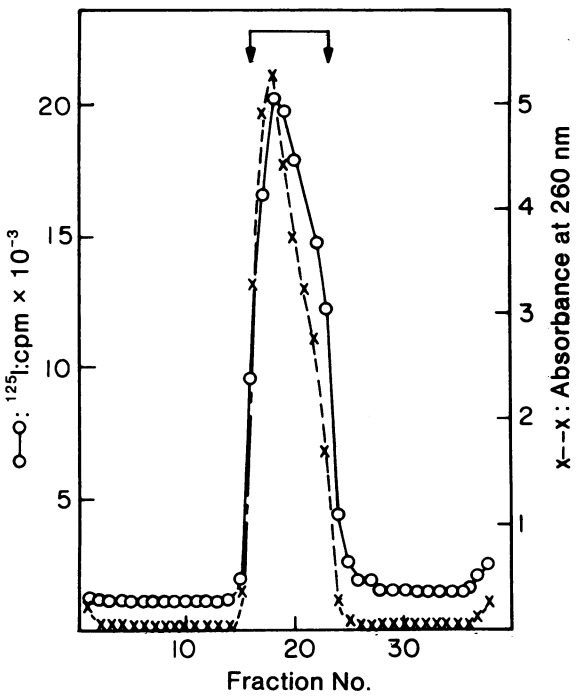

FIG. 2. Band profile of the protein–DNA complex in a guanidine·HCl–CsCl gradient. The protein–DNA complex was dissolved in 5 ml of 4 M guanidine·HCl–3 M CsCl–10 mM Tris·HCl–1 mM EDTA, adjusted to pH 7.0 with 0.1 M NaOH, and centrifuged for 64 hr at 40,000 rpm at 20° in an SW65 rotor. Forty percent of ^{125}I radioactivity banded in the middle of the gradient as one peak; the rest of the radioactivity appeared near the top. Peak fractions were collected and rebanded in the above solution. Fractions 16 through 23 (from the bottom), indicated as arrows, were pooled for analyses in Fig. 3. Recovery of input ^{125}I radioactivity was about 70%.

treated either with Pronase or with DNase I, then sedimented through the gradients (Fig. 1c). After treatment with Pronase or DNase I, the ^{125}I radioactivity was released from the fast sedimenting material and stayed at the top of the gradient. These results show that the ^{125}I radioactivity is in protein(s) that are attached to unit-length linear ss-DNA by an alkali-resistant bond.

In order to remove tightly associated adventitious proteins from the complex, we banded a portion of the ^{125}I-labeled complex in a gradient containing 4 M guanidine·HCl and 3 M CsCl. Forty to 46% of total ^{125}I radioactivity banded as one peak in the middle of the gradient; the rest of the radioactivity was at the top. On rebanding in guanidine·HCl–CsCl, all of the ^{125}I radioactivity remained associated with the DNA (Fig. 2). The center of the ^{125}I radioactivity profile was slightly displaced towards a lighter buoyant density from that of the absorbance profile. This result suggests that there are at least two DNA components in the population: DNA with a protein(s) and DNA without protein. However, it is not possible to estimate from this band profile what portion of the DNA contains protein.

A portion of the purified complex from the guanidine·HCl–CsCl gradient (Fig. 2, fractions 16 through 23) was incubated with DNase I. The treated and untreated samples were banded in neutral CsCl gradient. The results are shown in Fig. 3a. DNase treatment released the ^{125}I radioactivity from the DNA; the radioactivity now banded as a broad peak with a buoyant density of 1.35 g/cm^3. This observation is further evidence that the ^{125}I-labeled component is protein.

We believe that noncovalent bonds between a protein and a nucleic acid will be dissociated by alkali and/or by 4 M guanidine·HCl; therefore the observations reported above show that, in the present case, the protein is covalently attached to the DNA.

A portion of the purified complex (Fig. 3, fractions 16 through 23) was incubated at 37° with 3.86 M NH_2OH at pH 4.23 for 30 min or for 5 hr. As shown in Fig. 3b, NH_2OH treatment did not release the label from the DNA. Thus the protein-nucleic acid bond is not cleaved by hydroxylamine, which is known to cleave some phosphoamide bonds (6, 7).

Visualization of a protein dot on the end of linear ss-DNA

When a DNA sample from fraction 20 of the alkaline sucrose gradient (Fig. 1a) was prepared for EM by the BAC spreading method (5), a heavily stained dot attached to one end of linear ss-DNA was observed (Fig. 4a). Out of 70 full-length, traceable molecules, 15 were single-stranded circles and 55 were single-stranded linear molecules. Forty-three dots were observed on

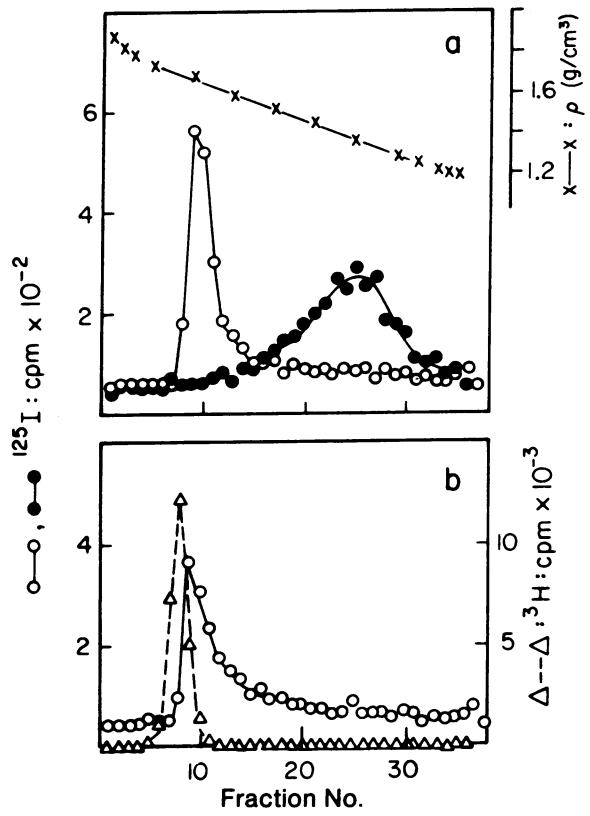

FIG. 3. The effects of DNase and hydroxylamine (NH_2OH) treatments on the buoyant density of the ^{125}I-labeled protein in CsCl density gradients. (a) Superimposed profiles of DNase I-treated and untreated samples from fractions 16–23, Fig. 2. (○) Untreated complex; (●) DNase I-treated sample. Sample was treated with DNase I (30 μg of DNase I in 0.13 ml of 10 mM Tris·HCl, pH 7.9–80 mM NaCl–10 mM $MgCl_2$) for 40 min at 37°, and reaction was stopped by addition of EDTA to 20 mM. (b) Superimposed profiles of NH_2OH-treated protein–SV40 DNA complex and PM2 [^3H]DNA. The samples in 3.86 M NH_2OH, pH 4.23, as described by Gumport and Lehman (7), were incubated at 37° for 5 hr and neutralized to pH 7.0 with concentrated KOH. (○) NH_2OH-treated protein-DNA complex; (△) NH_2OH-treated PM2 [^3H]DNA. The same results were obtained with a 30-min incubation time. The neutralized samples were adjusted to a density of 1.6 g/cm³ of CsCl in 10 mM Tris·HCl, pH 7.5–1 mM EDTA and a volume of 3 ml. The solution was layered between 3 ml of CsCl with a density of 1.4 g/cm³ and 2.5 ml with a density of 1.8 g/cm³; 2.5 ml of CsCl, 1.2 g/cm³, were layered on top to complete the step gradient. The tube was centrifuged for 50 hr at 20° at 38,000 rpm in an SW41 rotor. Fractions were collected from the bottom and examined for radioactivity. ^{125}I recoveries: (a) ○, 50%; ●, 35%; (b) ○, 50%.

32 linear molecules, and the distribution is shown in Fig. 4c. On molecules with one dot each, the distance to the closer end is presented as the relative position. When two or more dots were present on one DNA molecule, the dot closer to an end was assigned the smaller coordinate. There were no dots on the remaining 23 linear molecules nor on any of the 15 circular molecules. Twenty-nine out of the 43 dots observed were located at terminal positions. Four molecules had dots at both ends (Fig. 4b). However, the dot at one end was always larger and more prominent than the dot at the other end. When SV40 DNA that had been nicked by x-irradiation was similarly spread for EM, only three out of the 50 linear molecules seen had a terminal dot. This result indicates that a minimum of 45% of the nicked circular DNA contains protein and that the linkage between protein and DNA in the complex is stable in 98% formamide in low ionic strength.

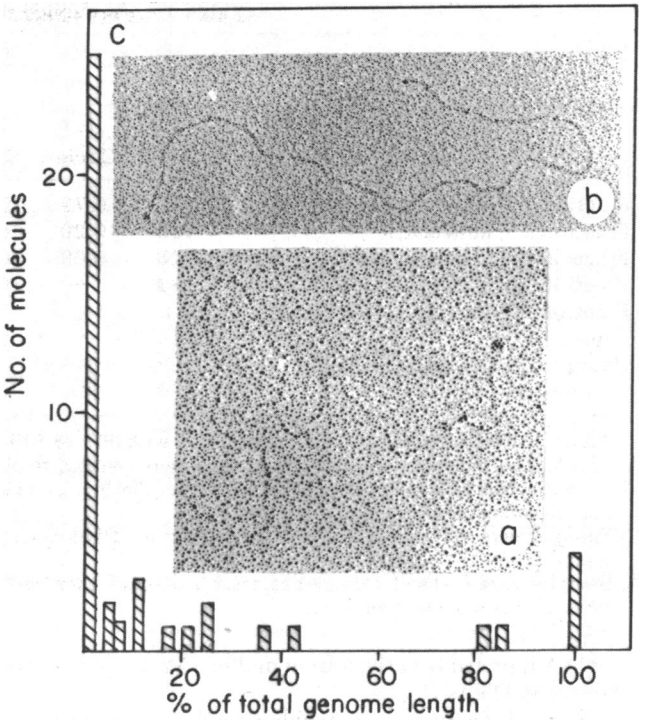

FIG. 4. Electron micrographs of linear ss-DNA with protein(s) and distribution of the position of the protein dots on DNA. (a) and (b) Electron micrographs of linear SV40 ss-DNA prepared by the BAC spreading technique. (a) Dot attached to one end; (b) dots probably at both ends. Photographs were taken at magnification of ×17,500. The figures show full-length linear ss-DNA. (c) The distribution of the position of the protein dots on linear SV40 ss-DNA.

Specificity of the nicks

We previously showed that the protein is attached at 0.67 map unit on SV40 DNA (1). We have now shown that it is frequently attached at one end of the linear ss-DNA resulting from dissociation of the nicked circles. We now wish to study the strand specificity of the nick and its relative position on the two strands. Representative fractions from an alkaline sucrose gradient containing mostly circular ss-DNA (71%, fraction 15, Fig. 1 and Table 1) were self-annealed and examined by EM in formamide-cytochrome *c* spreads. Examples of the molecules seen are shown in Fig. 5, and their frequency before and after annealing is listed in Table 1.

Several types of duplex DNAs were observed in the self-annealed fractions. After annealing, the predominantly single-stranded circular fraction 15 contained circular double-stranded DNA (ds-DNA) (39%), oligomeric ds-DNA (2%), partially renatured circular duplex DNA (38%) (Fig. 5b), and circular ss-DNA (20%). We attribute the formation of double-stranded circles mainly to reassociation of a circular single strand with a contaminating linear single strand. The partially renatured circles result from the reassociation of intact single-stranded circles. Because of the topological constraint, such circles cannot pair extensively. The average amount of apparent duplex was 490 ± 150 base pairs (Fig. 5 legend). The self-annealed linear fraction 20 contained circular ds-DNA (75%) (Fig. 5d), unit-length linear ds-DNA (8%) (Fig. 5d), oligomeric ds-DNA (13%) (Fig. 5e–h), and highly complexed ds-DNA (4%).

The extensive duplex formation in the two samples indicates that each fraction contains both strands. Therefore, the nick can occur on either strand of the DNA. (The highly complexed

Table 1. Frequency distributions of molecular forms

	Total no. counted	Fraction of molecules						Type of molecules		
		ss-Circle	ds-Circle	ss-Linear	ds-Linear	Oligomer	Complexed	b	i	j + k
Fraction 15, alkali-sucrose*	150	0.71	0.03	0.25	0.01	—	—	—	—	—
Fraction 15, annealed†	190	0.20	0.39	—	0.01	0.02	—	0.38	—	—
Fraction 20, alkali-sucrose*	125	0.09	0.10	0.79	0.02	—	—	—	—	—
Fraction 20, annealed†	183	—	0.75	—	0.08	0.13	0.04	—	—	—
Fraction 20, annealed/80% formamide‡	219	—	0.19	—	0.29	0.11	0.13	—	0.08	0.20
Fraction 26 (Pronase-treated), annealed†	193	—	0.61	—	0.13	0.17	0.10	—	—	—

Samples were prepared for EM as described with 50% or 80% formamide isodenaturing spreading conditions (14, 15) and with 0.5 μg/ml of DNA. The molecules on each photograph were counted to obtain frequency distributions. Fractions 15 and 20 are from the gradient in Fig. 1a; fraction 26 is from the gradient in Fig. 1c. The b, i, j, and k signify the types of molecules shown in Fig. 5.

* Samples were prepared shortly after isolation.

† Samples were self-annealed in 50% formamide at a DNA concentration of 5 μg/ml, in 0.1 M Tris·HCl, pH 8.4-10 mM EDTA for 1 hr at room temperature.

‡ Samples were first self-annealed as described above, then partially denatured in 80% formamide in 80 mM Tris·HCl, pH 8.4–8 mM EDTA for 10 min at room temperature.

ds-DNA is probably derived from multinucleation of linear and circular ss-DNAs.)

The high frequency of double-stranded forms in the annealed linear fraction did not change significantly when separated linear ss-DNA from a Pronase-treated sample (Fig. 1c, fraction 26) was self-annealed (Table 1). Therefore, the proteins attached to the end of ss-DNAs are not responsible for circularization and for oligomer formation. Several lines of evidence support the view that the nicks in the two strands are slightly staggered with respect to each other.

Occurrence of unit-length linear ds-DNA was noted in self-annealed fraction 20 (8%, Table 1). In some of the observed unit-length linear ds-DNAs (Fig. 5d), the two ends were located close to each other, suggesting that these DNA molecules were circular in solution, but that they contain cohesive ends that were dissociated by the spreading forces during mounting for EM.

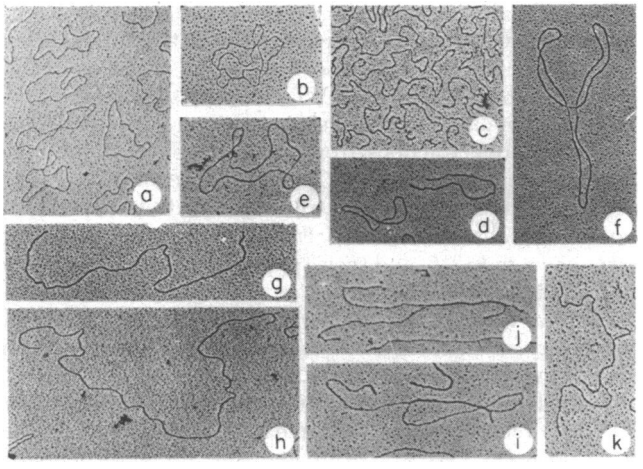

FIG. 5. Electron micrographs of types of DNA molecules observed in samples prepared as described in Table 1. (a) Circular ss-DNA in fraction 15, Fig. 1a. (b) Partially renatured circular DNA, self-annealed fraction 15. Apparent duplex regions in 30 such molecules were measured, and the extent of base pair formation was calculated as a ratio of the length of the apparent duplex segment in the molecules to the unit length of 5.2 kilobases of the circular double-stranded DNA (ds-DNA). Average length of such apparent duplex region is 490 ± 150 base pairs, on the assumption that the region is B-form DNA. This assumption is regarded as incomplete because these regions altogether must contain equivalent numbers of left- and right-handed turns. (c) Linear ss-DNAs in fraction 20, Fig. 1a. (d) Linear and circular ds-DNA, self-annealed fraction 20. (e–h) Circular-dimer (e) and trimer (f) and linear-dimer (g) and trimer (h), self-annealed fraction 20. (j–k) Partially denatured self-annealed DNAs.

Examples of oligomers from the annealed single-stranded linear fraction are shown in Fig. 5e–h. Most of these molecules appear to be entirely duplex with a length that is a multiple of the unit length. Of 122 oligomers traced and measured, there are 86 dimers, 24 trimers, 6 tetramers, 4 pentamers, and 2 hexamers. These molecules had very few observable single-strand branches. In a population of reassociated randomly nicked molecules, one would expect to see frequent single-strand branches. If there were any single-strand tails at the ends of the linear duplexes, they were too short to be reliably recognized and measured. This argues that the specific nicks in the two different strands must be staggered (in order to produce oligomers) but close to each other. Obviously, the staggered ends must be long enough so that the resulting duplexes are stable under the spreading conditions (50% formamide).

The high frequency of double-stranded circles in annealed fractions 20 and 26 also supports the staggered nick interpretation. Additional evidence in support of the specificity and staggered relation of the nicks comes from studies of the structure observed under more denaturing conditions (higher formamide spreads). Self-annealed fraction 20 was denatured in 80% formamide (80 mM Tris·HCl, pH 8.4–8 mM EDTA) at room temperature for 10 min and prepared for EM. Under this stronger denaturation condition, a decrease in appearance of circular ds-DNA (from 75% to 19%) was observed with simultaneous increase in appearance of unit-length linear ds-DNA (from 8% to 29%) and appearance of partially denatured forms (Fig. 5i, j, and k). Seventy-six percent of the observed molecules were monomeric duplex DNA or partially denatured DNAs (Table 1), and 24% of the molecules were partially denatured complex DNAs. Of monomeric DNAs, the frequency of circular ds-DNA, unit-length linear ds-DNA, partially denatured linear DNA, and partially denatured circular DNA was 0.25, 0.38, 0.27, and 0.1, respectively. Among the partially denatured linear DNA molecules composed of two unit-length complementary strands, 70% had two single-strand tails of approxi-

mately equal length attached to duplex segment (Fig. 5j) and 30% had single strands of unequal length at the two forks (Fig. 5k). The latter we attribute to reassociation with a randomly nicked strand. Among 36 molecules interpreted as resulting from reassociation of specifically nicked strands (Fig. 5j), the average length difference of the two single-strand tails is 109 ± 83 nucleotides. The relative frequency of molecules of the types shown in Fig. 5j and k indicates that the fraction of specifically nicked linear strands is between 70 and 85%. Molecules of the type shown in Fig. 5i are due to partial denaturation of a duplex formed from a linear and a (contaminating) circular single strand. The partial denaturation patterns (data not shown) are consistent with the hypothesis of a specific nick in the linear strand.

DISCUSSION

The experiments reported here demonstrate that in the protein–DNA complex, isolated from SV40, the protein is covalently attached to one end of the nicked ss-DNA, as visualized by the BAC spreading method, and the nicks occur on either strand, at specific sites, slightly staggered with respect to each other. A bond between protein and DNA is stable to $NaDodSO_4$, alkali, 4 M guanidine·HCl, 98% formamide, and 3.86 M NH_2OH (pH 4.23). Even though NH_2OH cleaves *E. coli* polynucleotide ligase–AMP complex (7), the insensitivity of protein–SV40 DNA complex to the reagent does not rule out the possible presence of a phosphoamide bond. The stability of such a bond has been reported to be influenced by adjacent peptide and nucleotide sequences (6).

The SV40 protein–DNA complex is found in the population of nicked circular DNA when viruses (in TD buffer) are treated with $NaDodSO_4$ and dithiothreitol. The presence of dithiothreitol is essential for both the introduction of nicks on the DNA and for the formation of the protein–DNA complex. Omission of dithiothreitol from our deproteinization procedure leads to a DNA population substantially devoid of nicks (unpublished data). Not all of the nicked DNA contains a protein, as indicated by the frequency of observation of a protein dot at the end of ss-DNA by the BAC method and in the experiment in which the displacement of the absorbance profile relative to the ^{125}I radioactivity profile in the guanidine·HCl–CsCl gradient was observed. However, most of the introduced nicks are specific. There are several possible explanations for the heterogeneity of protein content. (*i*) All nicked molecules contain proteins, but they are heterogeneous in molecular weight. Protein of low molecular weight could have escaped detection on DNA by the BAC method. (*ii*) A protein is attached to all DNAs when a nick is introduced and some of the proteins are removed. (*iii*) The nick is introduced by a site specific endonuclease. Another protein is then sometimes, but not always, covalently attached to the DNA.

The most frequent kind of full-length single-stranded linear molecule with attached dot(s) (presumably protein) seen by EM has a dot at one end only. A few molecules were seen with dots at both ends, although one was always larger than the other. We do not know at present whether attachment at two ends is due to some nonspecific interaction or is significant for the specific interactions.

We do not know the biological significance of the protein other than that the protein and nicks are located near the origin for replication and for transcription. Hamster antiserum against T antigen did not immunoprecipitate the substantially purified protein–DNA complex (unpublished data), but this does not rule out that the native protein is the T-antigen. The introduction of nicks at close but different locations on the two strands is intriguing. It seems that the protein(s) that nicks DNA recognizes specific sequence(s). If so, it implies that this sequence occurs in a reverse order on the two strands at different locations. Preliminary data suggest that a portion of the DNA in nicked circular DNA contains nicks on both strands of SV40 DNA.

Protein–DNA complexes with the protein covalently attached to DNA have been isolated from plasmids of *E. coli* (see ref. 8) and *Proteus mirabilis* (9) and from adenovirus (10–12). In the Col E_1 protein–DNA complex, the protein is attached to the 5′ terminus of the DNA (13). The SV40 protein–DNA complex is in some ways similar to that of relaxation complexes of bacterial plasmids. Nicks are introduced in closed circular DNA at a specific site, and protein-denaturing agents are required to form the complex. However, the nick is strand specific for plasmids (8, 9) but not for SV40.

We are indebted to Profs. J. Vinograd and N. Davidson, in whose laboratories this work was conducted, for advice, encouragement, and criticism. This research has been supported by National Institutes of Health Grants CA08014, GM20927, and GM10991.

1. Kasamatsu, H. & Wu, M. (1976) *Biochem. Biophys. Res. Commun.* **68**, 927–936.
2. Bolton, A. E. & Hunter, W. M. (1973) *Biochem. J.* **133**, 529–539.
3. Hirt, B. (1967) *J. Mol. Biol.* **26**, 365–369.
4. Sharp, P. A., Hsu, M.-T., Ohtsubo, E. & Davidson, N. (1972) *J. Mol. Biol.* **71**, 471–497.
5. Vollenweider, H. J., Sogo, J. M. & Koller, Th. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 83–87.
6. Shabarova, Z. A. (1970) in *Progress in Nucleic Acid Research and Molecular Biology*, eds. Davidson, J. N. & Cohn, W. E. (Academic Press, New York), Vol. 10, p. 145.
7. Gumport, R. I. & Lehman, I. R. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 2550–2563.
8. Helinski, D. R., Lovett, M. A., Williams, P. H., Katz, L., Kupersztoch-Portnoy, Y. M., Guiney, D. G. & Blair, D. G. (1975) in *Microbiology-1974* (American Society for Microbiology, Washington, D.C.), pp. 104–114.
9. Morris, C. F., Hershberger, C. L. & Rownd, R. (1973) *J. Bacteriol.* **114**, 300–308.
10. Robinson, A. J., Younghusband, H. B. & Bellett, A. J. D. (1973) *Virology* **56**, 54–69.
11. Robinson, A. J. & Bellett, A. J. D. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **39**, 523–531.
12. Brown, D. T., Westphal, M., Burlingham, B. T., Winterhoff, U. & Doerfler, W. (1975) *J. Virol.* **16**, 366–387.
13. Guiney, D. G. & Helinski, D. R. (1975) *J. Biol. Chem.* **250**, 8796–8803.
14. Davis, R. W., Simon, M. & Davidson, N. (1971) in *Methods in Enzymology*, eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. 21, p. 413.
15. Davis, R. W. & Hyman, R. W. (1971) *J. Mol. Biol.* **62**, 287–301.